CHAPTER 5

Bacteriology

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I. Introduction

This section defines the procedures and techniques used to correctly identify the target bacterial pathogens identified for the Survey (*Yersinia ruckeri*, *Aeromonas salmonicida* and *Edwardsiella ictaluri*). Proper identification relies on bacterial growth characteristics, appropriate biochemical tests, and corroboration by serological techniques.

Pathogens of Regional Importance (PRI) such as *Flavobacterium columnare* and *Flavobacterium psychrophilum* have special requirements for culture and serological confirmation. Several excellent sources are listed in the Reference section for identification of PRIs and other bacteria that may be isolated from fish sampled for the Survey. Additional media formulas are also provided for PRIs in Appendix A.

II. Media Preparation

A. PLATE MEDIA

1. Prepare media in stainless steel beakers or clean glassware according to manufacturer instructions. Check pH and adjust if necessary. Media must be boiled for one minute to completely dissolve agar. Common media recipes are given in Appendix A.

2. Cover beaker with foil, or pour into clean bottles being sure to leave lids loose. Sterilize according to manufacturer's instructions when given, or at 121°C for 15 minutes at 15 pounds pressure.

3. Cool media to 50°C.

4. Alternatively, media can be autoclaved and cooled to room temperature and refrigerated for later use. Store bottles labeled with media type, date and initials. When media is needed, boil, microwave or use a water bath to completely melt the agar. Cool to 50°C, then proceed to step 5.

5. Before pouring media, disinfect hood or counter thoroughly and place sterile petri dishes on the disinfected surface.

6. Label the bottom of each plate with medium type and date prepared.

7. Remove bottle cap and pour plates or dispense with a Cornwall pipet, lifting each petri dish lid as you go. Pour approximately 20 ml per 100×15mm petri dish. Replace lids as soon as the plate is poured.

8. Immediately wash medium bottle and cap in hot water to remove agar and clean up any spilled agar.

9. Invert plates when the media has cooled completely (~ 30-60 minutes) to prevent excessive moisture and subsequent condensation on the plate lid.

10. Allow plates to sit overnight at room temperature. Store plates upside down in the refrigerator in a tightly sealed plastic bag or plate storage tin.

11. Follow manufacturer's recommendation for storage period of prepared media.

B. TUBE MEDIA

1. Prepare media in stainless steel beakers or clean glassware according to manufacturer's instructions. If the medium contains agar, boil for one minute to completely dissolve the agar.

2. Media with indicators must be pH adjusted. This can be done when medium is at room temperature; otherwise compensation for temperature needs to be made.

3. Arrange test tubes in racks. Disposable screw cap tubes can be used for all tube media.

4. Use an automatic pipettor or Pipet-aid™ to dispense the medium. If using the Brewer or Cornwall pipet prime with deionized water, then pump the water out of the syringe prior to pipetting and discard the first few tubes of media that are dispensed. Dispense approximately 10 ml media in 16×125mm or 20×125mm tubes. Close caps loosely.

5. Immediately after use, rinse the automatic pipettor in hot tap water followed by distilled water to remove all media and prevent clogging of the instrument.

6. Follow manufacturer's recommendation for autoclave time and temperature.

7. If making slants, put tubes in slant racks after autoclaving. Adjust the slant angle to achieve the desired slant angle and butt length (i.e. short butt and long "fish-tail" slant for TSI or a standard slant over ¾ of the tube length for BHIA).

8. Tighten caps when tubes can be easily handled but still warm to the touch. Cool completely to room temperature in the slanted position.

9. Label the tubes or the tube rack with type of medium and date made.

10. Store at 2-8°C, following manufacturer's recommendation for period of long-term storage.

III. Media Formulations

Numerous differential media and biochemical tests can be used to determine identification of bacterial cultures. Some common bacteriological media are listed in Appendix A, but this list is incomplete and a good bacteriology media reference such as Difco Manual, McFaddins Biochemical Media Used for Detection of Bacteria, or Atlas's Handbook of Microbiological Media should be used for additional information. The purpose and use of various media are described in the testing section.

IV. Bacterial Culture Isolation

A. Aseptically inoculate samples onto BHIA tubes or plates.

B. Incubate for 24-48 hours at 22°C (room temperature). If no growth occurs at 24 and 48 hours, record this information on the data sheet. If no growth occurs after 72 hours, samples are discarded.

C. When growth does occur on field collection tubes or plates, use a sterile loop or needle to select a single colony to subculture onto fresh BHIA plates. If colonies are not well isolated, the plate will have to be re-inoculated on BHIA and thoroughly struck over the entire plate surface to achieve isolation of bacteria.

D. Incubate at 22°C for 24 hours to allow bacterial growth; all tests should be performed on 24-48 hour cultures.

    1. Using a sterile needle or small loop, pick individual distinct bacterial colonies. Assign an isolate number to each pure colony and record all colony characteristics on the data sheet.

    2. Begin initial testing to determine presumptive identification of pure strain bacterial cultures (CO, motility, catalase and Gram stain).

    3. Based on preliminary tests, follow the Flowchart (Appendix D) to determine which biochemical tests are needed to determine identification.

    4. Inoculate labeled biochemical tubes with isolate number, date, and initials. Follow the directions for interpretation of biochemical tests in the next section.

E. Treat all bacterial cultures as potential pathogens. When testing is complete, either cryopreserve isolates of interest, or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave.

V. Gram Stain

Gram stain pure strain cultures to determine whether Gram negative or Gram positive. Gram staining detects a fundamental difference in the cell wall composition of bacteria. (Kits are available commercially, or formulas for reagents are listed in Appendix B.)

A. Prepare a bacterial smear from a pure culture
    1. Put a drop of saline, distilled water, or PBS on a clean glass slide

    2. Using a sterile loop or needle touch an isolated colony and mix in the water drop.

    3. Mix until just slightly turbid (light inoculum is best, excess bacteria will not stain properly).

    4. Let air dry and heat fix. Do not overheat; slide should not be too hot to touch.

    5. Allow to cool.

B. Flood the slide with crystal violet, and allow to remain on the slide for 60 seconds

C. Wash off the crystal violet with running tap water.

D. Flood the slide with Gram's iodine, and allow to remain on the slide for 60 seconds.

E. Wash off with running tap water.

F. Decolorize with 95% alcohol and 5% acetone solution until the solvent flows colorless from the slide (approximate 5-10 seconds). Excessive decolorization should be avoided since it may result in a false gram-negative reading.

G. Rinse immediately with running tap water.

H. Counter stain with Safranin for 90 seconds.

I. Rinse with tap water and allow to air dry.

J. RESULTS:

    1. Gram negative: cells are decolorized by the alcohol-acetone solution and take on a pink to red color when counterstained with safranin.

    2. Gram positive: cells retain the crystal violet and remain purple to dark blue.

K. QUALITY CONTROL: Use a known bacterial culture as a control for each case history to assess correct staining and interpretation (cultures can be obtained from ATCC and maintained at 2-8°C for long term use)

1. Positive: *Staphylococcus sp.*

2. Negative: *Yersinia ruckeri*

VI. Presumptive Identification of Gram Negative Bacteria

Refer to Flow Chart in Appendix D and perform the following series of tests.

A. **Cytochrome oxidase** – this test determines the presence of cytochrome oxidase enzymes. The use of an iron-containing metal inoculation loop can lead to a false-positive reaction. Use only plastic or platinum loops for this test.

1. RESULTS:
   a. Positive: purple color within 5-10 seconds (reactions that occur after 10 seconds are negative).
   b. Negative: no purple color.

2. QUALITY CONTROL:
   a. Positive: *Pseudomonas sp.*
   b. Negative: *Yersinia sp.*

B. **Motility** – this test determines if a bacterial isolate is motile by means of flagella.

1. Place a drop of distilled water or sterile PBS onto the center of a clean microscope coverslip. Place an additional tiny drop in one corner of the coverslip (to adhere the coverslip to the depression slide when it is inverted). Inoculate the center drop from a pure strain culture that is 24-48 hours old using a sterile loop. Carefully invert the coverslip and place over the concave portion of a hanging drop slide. Observe for motility using phase contrast at 400× magnification on a compound microscope. Care should be taken to not interpret "drift" or "Brownian motion" as motility. Record results as motile or non-motile.

2. If this method fails to show motility then:
   a. Inoculate a nutrient broth with the isolate and incubate at room temperature until growth is obtained, usually 24 hours. After incubation use a sterile loop or sterile dropper and place a drop on a clean coverslip. Place a tiny drop of distilled water in one corner of the same coverslip. Continue as above.
   b. Semi-solid motility test medium can also be used. Stab the medium with a small amount of inoculum. Incubate overnight at room temperature. If the bacterial species is motile, the medium will become turbid with growth that radiates from the line of inoculum. If the bacterial species is non-motile, only the stab line will have visible bacterial growth.

C. Catalase – this test determines bacterial production of catalase enzymes.

1. Place a drop of hydrogen peroxide (3% H_2O_2 - reagent grade) on a microscope slide or in the concave surface of a hanging drop slide.

2. With a sterile loop, collect a sample of 18-24 hour old pure bacterial culture.

3. Place the loop in the hydrogen peroxide.

4. If the test is positive, there will be immediate bubbling or foaming, and liberation of O_2 gas.

5. Record results.

D. OF Basal – Bacteria metabolize carbohydrates by oxidative and/or fermentative pathways. Oxidation occurs in the presence of atmospheric oxygen (aerobic), whereas fermentation takes place in an anaerobic environment. Metabolism of the carbohydrate dextrose by either an aerobic or anaerobic pathway results in acid production. The resulting acidic environment causes the Brom Thymol blue pH indicator in the medium to turn from green to yellow. The presence of bubbles in the tube indicates gas production (aerogenic). If no reaction occurs, the medium can remain unchanged or become alkaline (blue at the surface).

1. A deep butt tube (~7 ml in 16 × 125mm or ~ 9 ml in 20 × 125 mm) is used for this test.

2. With a sterile needle take a small inoculum from an isolated colony and stab to the bottom of the tube.

3. Incubate at 22°C for 24-48 hours. Check tubes at 24 hr for acid and/or gas production.

4. RESULTS: A = acid; AG = acid + gas, N = no change or alkaline

	Top of Tube	Bottom of Tube
Oxidative	A	N
Fermentative	AG or A	AG or A
Non-reactive	N	N

5. QUALITY CONTROL: Fermentative: *Aeromonas* sp.
Oxidative: *Pseudomonas* sp.

E. Triple Sugar Iron (TSI) - This medium can determine the ability of an organism to utilize specific carbohydrates incorporated in a basal growth medium, with or without the production of gas, along with the determination of hydrogen sulfide (H_2S) production.

TSI agar contains the three sugars in varying concentrations: glucose (1X), lactose (10X), and sucrose (10X). It also contains the pH indicator phenol red. If sugar fermentation occurs, glucose will be initially used and the butt of the tube will be acidic (yellow). After glucose utilization the organism may continue to ferment the remaining sugars. If this occurs the entire tube will become acidic. Certain bacteria are unable to utilize any sugars and will breakdown the peptone present. Peptone utilization causes an alkaline (red) shift in the medium that causes a color change from orange to red. Blackened medium is caused by hydrogen sulfide production, which changes ferrous sulfate to ferrous sulfide. In addition, splitting of the medium or presence of bubbles in the butt of the tube can determine gas production.

1. With a sterile needle inoculate the TSI slant by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle is drawn out of the tube. Screw the cap on loosely.

2. Incubate at 22°C. Read after 18-24 hours.

3. RESULTS:

   A = Acid; K = Alkaline; H2S = Hydrogen sulfide produced; N =No change

Slant/Butt	Color	Interpretation
K/N or K/A	red/orange (oxidative) or red/yellow (fermentative)	only peptone utilized or only glucose-fermented
A/A	yellow/yellow	glucose, plus lactose and/or sucrose-fermented
gas	splitting or bubbles	gas production
H_2S	black butt	Hydrogen sulfide produced

4. QUALITY CONTROL: A/A: ± gas: *Aeromonas* spp., *Escherichia coli*
   K/A w/gas H_2S: *Edwardsiella tarda, Salmonella typhimurium*
   K/A: *Proteus spp., Shigella flexneri*
   K/N: *Pseudomonas spp.*
   K/N w/H_2S: *Pseudomonas putrafaciens*

F. O/129 Discs – this test determines the sensitivity of a bacterial organism to the vibriostatic agent 2,4-diamino-6,7 di-isopropylpteridine (O/129).

1. Suspend bacteria in sterile saline.

2. Streak suspension on plate in three planes with a cotton swab.

3. Aseptically place sensitivity disc in the center of inoculum.

4. Incubate at 22°C for 24 hours.

5. This test can be done on the same plate as the antibiotic sensitivity test.

6. RESULTS: Sensitive: Zone of inhibition around disc
   Resistant: Growth adjacent to disc

7. QUALITY CONTROL: Positive: *Vibrio anguillarum*
   Negative: *Aeromonas hydrophila*

G. Commercial Identification Systems – Several commercial test strips or kits are available for biochemical testing of bacteria. Bear in mind that these kits are designed for human and/or animal testing and the manufacturer's recommended incubation temperature is 37°C. The decreased incubation temperature (22°C- room temperature) required for most fish pathogens results in slightly different reactions and longer incubation periods. Therefore, test results may not follow the manufacturer's identification profiles exactly. A good approach to this problem is to develop a library of known fish pathogen profiles based on serological testing. Referring to this information will assist with interpretation when these commercial test strips are used at room temperature (see Appendix E for some common API profiles based on known bacterial isolates).

1. API 20E™ – The API 20E™ system is a standardized, miniaturized version of conventional procedures for the identification of *Enterobacteriaceae* and other Gram-negative bacteria. It is a ready-to-use, microtube system designed for the performance of 23 standard biochemical tests from isolated colonies on plating medium. The test strip consists of microtubes containing dehydrated substances which are reconstituted by adding a bacterial suspension, incubated so that the organisms react with the contents of the tubes, and read when the various indicator systems are affected by the metabolites or added reagents, generally after 18-24 hours of incubation. Refer to the instructions enclosed with each kit for more detailed information. The API system™ is available from bioMérieux (1-800-638-4835, catalog #20-109/20-179).

2. Bionor™ – There are three Bionor Aqua™ rapid agglutination tests that are useful in the fish pathology labs; Mono-As™ for *Aeromonas salmonicida* (product # DD 020), Mono-Va™ for *Vibrio anguillarum* (product # DE 020), and Mono-Yr™ for *Yersinia ruckeri* Type I (product # DC 020). These kits contain a test reagent and a control reagent. The test reagents consist of mono-dispersed particles coated with antibodies that form a granular particle agglutination pattern when mixed with the homologous bacteria. When the bacterial isolate is mixed with the control reagent, no agglutination will appear. See instructions enclosed with each test for complete directions. These tests appear to be very specific. For example, the Mono-As will not agglutinate with *Aeromonas hydrophila* and the Mono-Yr™ is type I specific. The test kits can be purchased from Bionor, Strømdaljordet 4, P.O. Box 1868 Gulset, N-3701 Skien, Norway.

3. Minitek™ – the Minitek system is no longer available from the manufacturer, Becton Dickinson & Company.

4. Biolog – MicroLog™ is a microbial identification system able to identify and characterize a wide variety of organisms based on carbon source utilization. The system has identification databases that contain over 1400 different species/genera of aerobic and anaerobic bacteria and yeasts. The identification databases include a wide variety of organism including animal, plant, and water pathogens. The system also allows the user the capability to build customized organism databases. All organisms can be identified/characterized using one of four standardized MicroPlates™; GN2-MicroPlate™ for gram-negative bacteria; GP2- MicroPlate™ for gram-positive bacteria; AN-MicroPlate™ for anaerobic bacteria; and YT-MicroPlate™ for yeasts. Each of these MicroPlates™ contains a pre-selected group of carbon sources as well as an indicator dye. When the appropriate MicroPlate™ is inoculated and incubated, a characteristic pattern (fingerprint) of the organism develops based on which carbon sources the organism can utilize. The resulting pattern can be read either visually or with Biolog's MicroStation Reader™. The results are then compared by the system software to the extensive organism database for final identification. Biolog sells both manual and automated versions of its popular identification/characterization system. Products are available directly from Biolog, 3938 Trust Way, Hayward, CA. 94545 (1-510-785-2564 or website www.biolog.com).

VII. Characteristics of Target Bacterial Pathogens

A. *Aeromonas salmonicida*: Gram-negative small rod, non-motile, brown diffusible pigment* on TSA or BHIA, cytochrome oxidase positive**, ferments OF basal glucose. Additional biochemical testing on API and corroboration by serological methods (agglutination or FAT) is recommended.

B. *Edwardsiella ictaluri*: Gram-negative small rod, motile, cytochrome oxidase negative, ferments OF basal glucose, produces alkaline slant and acid butt with gas (K/Ag) on TSI. Further biochemical testing on API, and serological testing for corroboration is recommended.

C. *Yersinia ruckeri*: Gram-negative small rod, motile, cytochrome oxidase negative, alkaline/acid (K/A) on TSI, ferments OF basal glucose. Biochemical tests with API and corroboration by serological testing are recommended.

D. *Renibacterium salmoninarum*: Gram-positive rod, extremely slow growing (4-6 weeks) on KDM-2 or specialized growth agar. Identify with FAT or ELISA. Corroboration with PCR.

* Some strains of *A.salmonicida* do not produce brown diffusible pigment, or pigment production is delayed.

** An Oxidase-negative *A.salmonicida* has been reported (Chapman *et al.,* 1991)

VIII. Corroboration Methods

A. Slide Agglutination Test - Slide, or serum agglutination, test confirms bacterial identification by agglutination of a pure bacterial culture with its specific antiserum (e.g., *Aeromonas salmonicida*, *Yersinia ruckeri, Edwardsiella ictaluri as well as PRI's such as Flavobacterium psychrophilum* and *Flavobacterium columnare*).

1. Procedure**:**

   a. Put 0.5-1.0 ml PBS into a 12x75mm tube. (**A. salmonicida* may work better with deionized water.)
   b. Sterilize a loop and collect a sample of a 24-48 hour pure bacterial culture.
   c. Suspend the bacteria in the PBS by thoroughly vortexing.
   d. Heat *F. columnare* and *F. psychrophilum* cultures 5 minutes at 50-55°C to prevent auto agglutination.
   e. Put one drop of bacterial suspension onto each of three wells of a ring plate.
   f. Negative Control - on the first well, place a drop of PBS (*or water).
   g. On the second well, place a drop of antiserum for the appropriate bacterium, and on the third well, place a drop of normal rabbit serum (NRS).
   h. Mix the drops together by gently rocking the plate back and forth. You can also use a rotating plate set on slow speed.
   i. Allow 5-10 minutes for agglutination to occur. It may be helpful to observe the plate on a light stand or with an illuminated magnifier to see agglutination of bacteria and antibody.
   j. Interpretation: Agglutination in the well with bacteria and antiserum is a positive test; it is often referred to as a somewhat flaky 'dissolved aspirin' appearance. Negative controls should appear turbid, but without agglutination.

B. Fluorescent Antibody Technique (FAT) for bacteria. The Fluorescent Antibody Test is one serological method for corroboration testing of Gram-negative bacterial isolates such as *Aeromonas salmonicida* and *Yersinia ruckeri*. It is also often used for the Gram-positive bacterium *Renibacterium salmoninarum* (Rs), the causative agent of bacterial kidney disease (BKD) in very small fish where the quantity of kidney tissue is inadequate for ELISA.

In general, there are two types of staining procedures that utilize fluorescent antibodies; the indirect (IFAT) and direct (DFAT) techniques. The principle and techniques are similar, however the indirect utilizes a second antibody, which is often biotinylated for increased sensitivity. The direct FAT is used more commonly for bacterial corroboration testing and staining of *Renibacterium salmoninarum*. The indirect method, IFAT, is most often used for corroboration testing of viral isolates by staining cell cultures infected with virus. This section describes the DFAT only; see Chapter 12- Corroboration Testing of Viral Isolates, for the IFAT protocol.

There are three basic steps for DFAT: preparing and fixing bacterial cultures or kidney tissue on multiple-well glass slides; staining the slides with antibody reagents; reading and interpreting the slides.

1. Preparing the slides (2 methods described here for pure bacteria or tissues):

    a. Pure Bacterial Cultures (corroboration testing of pure isolates of *Aeromonas salmonicida* or *Yersinia ruckeri*). Pure isolates of bacteria are diluted in sterile PBS and applied to 2 replicate wells of an FAT slide. Air-dry and fix in absolute methanol for 5-10 minutes. Proceed to step #2.
    b. Kidney Smears from small fish - DFAT for *Renibacterium salmoninarum.* Kidney samples for FAT most often are collected in the field during necropsy. Kidney tissue is collected from each fish using sterile, individual tools to avoid cross-contamination between samples. The kidney tissue can be blotted or squashed onto the well area of the slide however the smear should be thin (thick smears tend to adhere poorly to the slide and are difficult to view microscopically).
    c. After the tissue has completely dried, slides are fixed in methanol for approximately 5-10 minutes.
    d. At the lab (or receiving facility), the slides are checked for completeness of labeling (case number and fish identification) and then stained immediately or refrigerated for staining on a subsequent day.

2. Staining - Once slides are prepared, the staining procedure is the same regardless of the sample type (bacterial isolate or kidney tissue), however a counter stain is usually not required for bacterial cultures.

    a. Positive and Negative Controls: Slides containing both a non-cross-reacting bacterial species and a known positive control can be prepared in quantity and stored refrigerated for use as controls for DFAT staining. Positive controls are always used in corroboration testing to correctly identify morphology and fluorescence of the bacteria in question. The negative control is important in determining overall staining technique, background debris, and non-specific fluorescence.
    b. Place slides in dark, humidified chamber, and place one drop of specific FITC conjugate on each well of the sample slide and control slides.
    c. Incubate for 60 minutes at room temperature.
    d. Using a squirt bottle or transfer pipet and GENTLY rinse the slides with PBS (or FTA Buffer) by flooding the solution over all wells.
    e. Place the slides in a staining rack and GENTLY rinse in the following order:
        i. PBS for 30 seconds
        ii. Evan's blue counter stain for 3-4 minutes for *Renibacterium salmoninarum*. (A counter stain is not necessary for corroboration testing of pure bacterial cultures, although Rhodamine can be used if desired.)

iii. Final rinse/soak in PBS or FTA buffer for 5-10 minutes.

f. Air-dry completely.

g. Add a very small drop of FA Mounting Fluid, pH 9 (Difco #3340-57) to each well. Place a 24x50 mm coverslip over the slide using care not to trap air bubbles.

h. Spread the mounting fluid by gently pressing the coverslip with the blunt end of a pen or lab marker in the spaces between the individual wells. The fluid should just cover each individual well (If the mounting medium extends into other wells, or spreads out from under the coverslip, too much mounting fluid was applied).

i. Add one drop of immersion oil to the coverslip over every other well and examine at 1000X using the epiflourescent filter.

3. Reading and Rating: Slides are read at 1000x on a compound fluorescence microscope (refer to the microscope manufacturer for correct wavelength and filters required for FITC epifluoresence microscopy). The positive and negative control slides are read first. This has two purposes: (1) quality assurance for the staining process (positive control should have myriad numbers of fluorescing bacteria, the negative control should have no fluorescence); and (2) to familiarize the reader with the correct bacterial size, shape, and magnitude of the fluorescent halo of bacteria in the positive control. The reader can refer back to the positive control as a reference, if needed, to confirm suspect bacteria in the sample wells.

   a. Bacterial Corroboration Testing: Positive bacterial isolates will fluoresce strongly and have the same morphology and size as the positive control.

   b. Kidney smears tested for *Renibacterium salmoninarum* - bacterium will have a distinctly **apple-green fluorescent cell wall**; be the appropriate **size - 1 micron long by 0.5 micron**; and be the **proper shape (bean shaped or pear shaped with one end appearing slightly pinched)**. Compare any suspect bacteria to the control slide to be sure all three of the above criteria are met for *Renibacterium salmoninarum*.

4. Hints for Good Results:

   a. Use FITC conjugates at optimum working dilution. Follow manufacturer's recommendation to test for optimum working concentration (see page 15).

   b. Filter all conjugated antibody reagents (.45um filter) prior to use to reduce background debris that fluoresce nonspecifically and cause difficulty in reading and interpreting the slides.

   c. Prepare thin smears; thick smears will not stain fix properly and are more easily washed off during the staining process, and thick slides require frequent focusing to observe multiple focal planes.

   d. Evenly distribute the kidney material in PBS, or use a very light inoculum of pure bacterial culture for each well (excess bacteria will stain poorly).

e. Heat-fix slides prior to fixing in methanol. If there is not an adequate way available to heat-fix the slides they can be air-dried and sent to the lab without fixation.
f. Control slides should be rinsed in separate Coplin jars to avoid any potential for cross contamination during the staining process.
g. Use anhydrous methanol to fix slides; the methanol reduces the lipid content of the preparation (de-fatting) increasing the overall fluorescence quality and intensity.

5. Fluorescent Antibody Technique Rating Guide (Alaska Department of Fish and Game - Pathology Laboratory Section Manual): Standard rating criteria for interpretation of the FAT on tissue smears based upon a minimum of 60 fields examined at 1000x.

a. **Negative (-)** No organisms seen in thirty fields examined.
b. **Plus/minus (±)** Organisms observed with questionable fluorescence or morphology not typical of the target organisms. Total of one typical organism observed but suspected of not originating from the sample examined, i.e., wash over from a high-level positive sample.
c. **One plus (1+)** One to five organisms observed. If only one organism is found, examination of up to 100 fields continues in an attempt to find a second organism that would confirm the 1+ status. If no other organism is detected the final +/- or 1+ interpretation is at the discretion of the individual reader.
d. **Two plus (2+)** Six to 50 organisms observed in which some fields will be negative and some will typically contain several organisms.
e. **Three plus (3+)** Fifty-one to 150 organisms observed with a typical field containing a dozen or more organisms.
f. **Four plus (4+)** Greater than 150 organisms with no more than 200 organisms in an average field.
g. **Clinical (C5+)** Greater than 200 organisms in an average field. Gross lesions are likely to be observed in the sampled kidneys from this category.

6. <u>FAT Material Suppliers</u>

J. Melvin Freed, Inc. (10-well slides)
Perkasie, PA 18944-0175
(215) 257-4344 P.O. Box 648

Erie Scientific Cel-Line Associates
(multi-well slides)
Newfield, NJ 08344
1-800-662-0973

ICN Molecular Biology (conjugates)
Costa Mesa, CA
1-800-854-0530

7. Reagents

Phosphate buffered saline, pH 7.2 (PBS)

NaCl	7.20 g
(sodium chloride, MW 58.44)	
Na_2HPO_4	1.48 g
(sodium phosphate, anhydrous dibasic, MW 141.96)	
KH_2PO_4	0.43 g
(potassium phosphate, anhydrous, monobasic, MW 136.1)	

Bring components to 1 L with distilled water. Adjust pH to 7.2 with 1 M NaOH or HCl.

8. Determination of Antiserum and Conjugate Working Dilutions for FAT.
In most cases commercially prepared antisera and conjugates are lyophilized in a concentrated state. Each should be reconstituted according to the manufacturer's instructions. Aliquots of 0.5 ml can be frozen for later dilution into a working solution of the reagent. Reagents are more stable if frozen as a stock solution. The proper working dilution is the highest dilution that still maintains maximum brightness of the fluorochrome. Generally the manufacturer will recommend between 1:20 to 1:50 using a suitable buffer (PBS) as the diluent. However, in all cases each laboratory must establish the proper working dilution by starting with the manufacturer's recommendation and bracketing, or testing dilutions on either side of the recommended concentration. The following example shows preparation of a direct FAT conjugate where the manufacturer recommends a working dilution of 1:40.

   a. Using the stock solution dilute the antiserum at 1:20, 1:30, 1:40, 1:50, 1:60 using PBS or another buffer as recommended.
   b. The FAT is performed on replicates of a known positive control, each replicate using a different dilution of the conjugated antiserum. In this way the working dilution can be determined as the endpoint of optimum fluorescence (the highest dilution that still provides a bright specific fluorescence with little or no background staining).

IX. Antibiotic Sensitivity Testing

When target bacterial pathogens are detected in the Survey, antibiotic sensitivity testing should be completed. While bacterial strains from natural populations of fish are not expected to have drug resistance to widely used antibiotics, this information should be verified by routinely performing drug sensitivity testing on all isolates of target fish pathogens. This information will help develop existing knowledge of drug resistance in aquatic environments.

The antibiotic sensitivity test determines the sensitivity or resistance of a bacterial isolate to specific antibiotics. Filter paper discs, each saturated with a different antibiotic, are evenly spaced on an agar plate surface inoculated with a lawn of the bacterial isolate to be tested. The

antibiotics diffuse into the surrounding medium, and create a decreasing gradient of the antibiotic concentration. If sensitive, a zone of bacterial growth inhibition (clear zone) will be present around the antibiotic disc. The following antibiotics can be routinely tested: oxytetracycline, sulfadimethoxine with ormetaprim (Romet 30), erythromycin, penicillin, polymyxin, O/129, and novobiocin.

1. Suspend cells from a pure bacterial culture in log phase (24-48 hour culture) in sterile saline to obtain a turbidity equivalent to a 0.5 McFarland standard.

2. Streak a Mueller-Hinton agar plate with a sterile cotton swab soaked with the bacterial suspension. Swab the plate in three separate planes.

3. Aseptically place antibiotic discs to be tested onto a freshly inoculated plate. Press onto agar surface lightly.

4. Invert the plates and incubate at 25°C for 24-48 hours. Observe and record results by measuring the diameters of the zone of inhibition around each disc.

5. Results: The following table lists zone of inhibition for each compound

   Sensitive: A specific diameter zone of inhibition around the disc.
   Resistant: Bacterial growth within the zone of inhibition or adjacent to the disc.

Zone Diameter Standards

Antimicrobial	Disc Content	Resistant	Intermediate	Sensitive
Erythromycin [1]	15ug	No zone	< 15 mm	≥ 15 mm
Novobiocin [2]	30 ug	No zone	< 10 mm	≥ 10mm
Oxolinic Acid [1]	2 ug	No zone	< 15 mm	≥ 15 mm
Oxytetracycline [1]	30 ug	No zone	< 15 mm	≥ 15 mm
Penicillin G [3]	10 U	≤ 11 mm	12-21 mm	≥ 22 mm
Romet 30 [1]	25 ug	No zone	< 15 mm	≥ 15 mm
O/129 [2]	0.1% (W/V)	No zone	< 7 mm	≥ 7 mm

[1] Model Comprehensive Fish Health Protection Program. Pacific Northwest Fish Health Protection Committee, September 1989.

[2] CFDM pathology case materials

[3] "Performance Standards for Antimicrobial Disc Susceptibility Tests," NCCLS 1981.

6. QUALITY CONTROL

Control cultures should be included with each sensitivity test. Cultures are available from American Type Culture Collection (ATCC) or National Collection of Industrial, Food and Marine Bacteria (NCIMB) of the United Kingdom (www/ncimb.co.uk.)

An excellent reference for antibiotic sensitivity testing is the Provisional Antimicrobial Susceptibility Testing Protocols – 27 December 1998, produced by the Workshop on MIC Methodologies in Aquaculture. Jerry Tjernagel (www/gtbugs@mnic.net) of MicroBiologics is attempting to make all the NCIMB strains available in the United States for use by fish health professionals. Also contact Tom Bell, USFWS – Division of Hatcheries, Washington Office for a copy of the Provisional Antimicrobial Susceptibility Testing Protocols or further information.

X. References

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Appendix A - Media Formulations

1. COMMON GROWTH MEDIA

A. Brain Heart Infusion Agar (BHIA) - A basic agar for most bacterial cultures, better for warm water fish.

- Suspend 52 g of dehydrated medium (Difco #0418) in 1 L distilled water and heat to boiling. Boil for one minute to completely dissolve agar. Sterilize and dispense as required.

B. Tryptic Soy Agar (TSA) – A basic agar for most bacterial cultures.

- Suspend 40 g of dehydrated medium (Difco #0369) in 1L distilled water and heat to boiling. Boil for one minute to completely dissolve agar. Sterilize and dispense as required. Store at 2-8°C. Final pH = 7.3±0.2 at 25°C.

C. Tryptic Yeast Extract Salt Agar (TYES) – A nutrient poor agar to help select for *Flavobacterium.*

Tryptone	5.0g
Yeast Extract	0.4g
$MgSO_4$ $7H_2O$	0.5g
$CaCl_2$ $2H_2O$	0.2g
Agar	10.0g
dH_2O	1 L

Mix ingredients and heat to dissolve completely. Sterilize and dispense as required. Store at 2.8°C. Final pH = 7.1±0.2.

D. Cytophaga Agar – This agar is best for isolating Flavobacterium and should be used when Cold Water Disease is suspected. Differences in cell and colony morphology aid in distinguishing the numerous bacteria that grow on this partially selective agar. Streak for isolation on two plates, incubate one plate at 20°C and the other at 25°C for 3-5 days.

RESULTS:

i. *Flavobacterium psychrophilum* – No growth at 25°C. Growth of bright yellow colonies at 20°C with convex center, and spreading periphery.
ii. *Flexibacter* (marine) – Orange or yellow colonies with uneven edges.
iii. *Flavobacterium columnare* – Light or no growth at 20°C. Greater growth of yellow convoluted centered colonies with rhizoid edges at 25°C.
iv. *Flavobacterium sp.* – Growth range 10-25°C, with best growth at 18°C. Light yellow round colonies, transparent and smooth.

FORMULA:

Tryptone	0.5g
Yeast Extract	0.5g
Sodium Acetate	0.2g
Beef Extract	0.2g
Agar	10.0g
dH_2O	1 L

Suspend the above ingredients in distilled water and heat to dissolve. Adjust pH to 7.2-7.4. Sterilize and dispense as required. Store at 2-8°C.

E. Nutrient Broth - To use when a liquid growth medium is preferred.
- Dissolve 8 g of the dehydrated medium (Difco #0003) in 1 L of distilled water. Dispense 5-7 ml/tube and autoclave for 15 minutes at 15 pounds pressure. Final pH = 6.8 at 25°C. Store at 2-8°C.

2. COMMONLY USED MEDIA TO IDENTIFY GROWTH AND BIOCHEMICAL CHARACTERISTICS

A. Motility test medium
- Suspend 20 g of dehydrated medium (Difco #0105) in 1 L distilled water and heat to boiling. Dispense 5 ml/tube and autoclave at 15 pounds pressure for 15 minutes. Cool medium in an upright position in a cold water bath. Final pH = 7.2 $\pm$ 0.2 at 25°C. Store at 2-8°C.

B. Cytochrome Oxidase Spot Test
- Individual test strips can be purchased from Remel (1-800-255-6730, catalog #38-191), or can be prepared in the laboratory with the following procedure.
- Cut Whatman #1 filter paper into strips and autoclave
- Spread filter paper on a glass petri dish.
- Put a few drops of oxidase reagent on each strip to saturate it.
  - Oxidase reagent: Tetramethyl-p-phenylenediamine dihydrochloride (Eastman Organic Chemicals) Prepare 1% aqueous solution.
  -
- Dry completely, then store in brown bottle. Freeze surplus until needed.

C. Triple sugar iron agar (TSI)
- Suspend 65 g of the dehydrated medium (Difco #0265) in 1 L of distilled water and heat to boiling. Dispense 7 ml/tube. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool in slanted position. Store at 2-8°C. Final pH = 7.4 at 25°C.

D. Oxidation/Fermentation (OF) medium
- Suspend 9.4 g of dehydrated medium (Difco #0688) in 1 L of distilled water and heat to boiling. Distribute in 100-ml amounts, autoclave for 15 minutes at 15 pounds pressure (121°C). To prepare final medium aseptically add 10 ml of a filter-sterilized (0.45µm) 10% glucose solution. Mix flask thoroughly and aseptically dispense 5.0 ml into sterile tubes. Store at 2-8°C. Final pH = 6.8 $\pm$ 0.2 at 25°C.

E. Mueller-Hinton agar
- Suspend 38 g of dehydrated medium (Difco #0252) into 1 L distilled water and heat to boiling to dissolve completely. Autoclave for 15 minutes at 15 pounds pressure (121°C). Aseptically dispense into sterile petri dishes. Store at 2-8°C.

Final pH = 7.3 $\pm$ 0.1 at 25°C.

F. MacConkey agar (for use with API)

- Suspend 50 g of the dehydrated medium (Difco #0075) in 1 L distilled water and heat to boiling. Dispense into bottles and autoclave at 15 pounds pressure for 15 minutes. Pour into petri plates. Final pH = 7.1 ± 0.2. Store at 2-8°C.

Appendix B - Reagents

A. Gram stain reagents

These stains can be ordered as a complete kit from VWR (#l5204-004) or can be reconstituted as follows:

Crystal violet

crystal violet (90% dye content)	20.0 g
ethanol (95%)	200 ml
ammonium oxalate	8.0 g
dH_20	800 ml

Mix first two ingredients and let sit overnight or until dye goes into solution. Add remaining ingredients and filter before use.

Gram's iodine

iodine crystals	1.0 g
potassium iodide	2.0 g
dH_20	300 ml

Decolorizer

acetone	40 ml
ethanol (95%)	60 ml

Safranin

safranin O	2.5 g
95% ethanol	100 ml
dH_20	900 ml

Filter safranin solution before use.

B. Oxidase reagent

Tetramethyl-P-Phenylenediamine Dihydrochloride	1.0g
dH_2O	100 ml

C. O/129 Discs

0.1% solution of pteridine dissolved in acetone

OR

0.1% solution of pteridine phosphate dissolved in sterile distilled water.

Autoclave at 15 pounds per square inch (psi) for 15 minutes. Place sterile blank discs (Difco, Bacto Concentration Discs ¼") in pteridine or pteridine phosphate solution, remove and dry at 37°C. Store at 2-8°C in a dark screw-cap bottle. Use on agar plates (usually Mueller-Hinton) to test the sensitivity of cultures to this vibrio-static agent.

Appendix C - Profiles Obtained with API-20E for Known Fish Pathogens

A. Yersinia ruckeri

The following table represents API20E profiles for *Yersinia ruckeri* when cultures were tested at 22°C rather than the manufacturer's recommended incubation temperature of 35-37°C. All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT. Based on the profile submitted to API, bacterial identification is given in order of probability, then remarks as to the likelihood of the profile are provided when profiles are poorly matched to the manufacturer's database.

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #1- Type II (11.29) 2. Nisqually Fall chinook (3/88)	**5307500**	1.) *Serratia mercescens* 2.) *Serratia liquefaciens* 3.) *Hafnia alvei*
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #2- Type II (11.29) 2. Fall Chinook, Suquamish R, WA (3/88) 3. Spring Chinook, Skookum Creek, WA (2/88)	**5107500**	(Same ID as 5307500 above) 1.) *Serratia mercescens* 2.) *Serratia liquefaciens* 3.) *Hafnia alvei*
Unknown source – Isolate confirmed by biochemical and serological testing.	**5144100**	1.) *Escherichia coli* 2.) *Yersinia ruckeri*
Eastern Fishery Disease Laboratory (EFDL) Positive Control - Type I (11.4)	**5107100**	"Unacceptable profile"
Coho, Quinault River, WA	**5106100**	"Questionable ID"
Late Fall Chinook, Battle Creek, CA (11/94)	**5105100**	1.) *Hafnia alvei* "Acceptable ID"
Notes from ERM archived files – previous testing	**5104500**	"Questionable ID"

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
1. Hagerman – Type I (11.4)	**5104100**	1.) *Yersinia ruckeri* "Very good ID"
Coho, Quilcene R., WA (11/88)	**5104000**	1.) *Yersinia ruckeri* "Very good ID"
Unknown source	**5100100**	1.) *Yersinia ruckeri* "Excellent ID"
Unknown source	**4105100**	1.) *Hafnia alvei*
Unknown source	**4104100**	1) *Yersinia ruckeri* 2) *Salmonella gallinarum*
Unknown source	**4104000**	1.) *Yersinia ruckeri* "Acceptable ID"
Unknown source	**0104100**	1.) *Yersinia ruckeri* "Acceptable ID"

*** *Yersinia ruckeri* generally fails to produce a positive citrate reaction when incubated at room temperature (22-25C). Refer to the API Manual for specific biochemical tests and interpretation of API20E™ profiles. Also see references listed on page 5-27.**

B. ***Aeromonas salmonicida***

The following represents API20E profiles for *Aeromonas salmonicida* isolates following manufacturer's instructions but incubating test strips at room temperature (22°C). All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT.

Bacterial Isolate ID (collection date)	**API PROFILE***	**Computer/Manual ID**
spp not identified, Makah NFH, WA (8/88)	**0006104**	1.) *Pseudomonas pseudomaleae* "Acceptable ID"
Winter Steelhead, Makah NFH, WA (1/89) Chum, Makah NFH , WA (12/89)	0006104	Same as above
Winter Steelhead, Quinault NFH, WA (1/89)	0006104	Same as above
Spring Chinook, Entiat NFH, WA (8/89)	0006104	Same as above
Spring Chinook, Quilcene NFH, WA (3/91)	**2006104**	1.) *Aeromonas salmonicida*
Profiles given in **API MANUAL** for *Aeromonas salmonicida*	**6006104** **6006504** **4006104** **2006104**	*Aeromonas salmonicida* "Good to Excellent ID"

***Aeromonas salmonicida* generally fails to produce positive relations for ONPG, ADH, and LDC when incubated at room temperature (22-25C).**

Profiles provided in the API Manual are based on positive reactions for some or all of these first 3 biochemical tests, therefore the first digit of the "acceptable" profiles for A.sal include the values 2,4, or 6. More often, a zero value is obtained after 24-48 hours incubation at room temperature. Longer incubation periods are required for these tests.

References:

Romalde, J.L., and A.E. Toranzo. 1991. Evaluation of the API-20E system for the routine identification of the enteric redmouth disease. Bull. Eur. Ass. Pathol. 11(4), 147.

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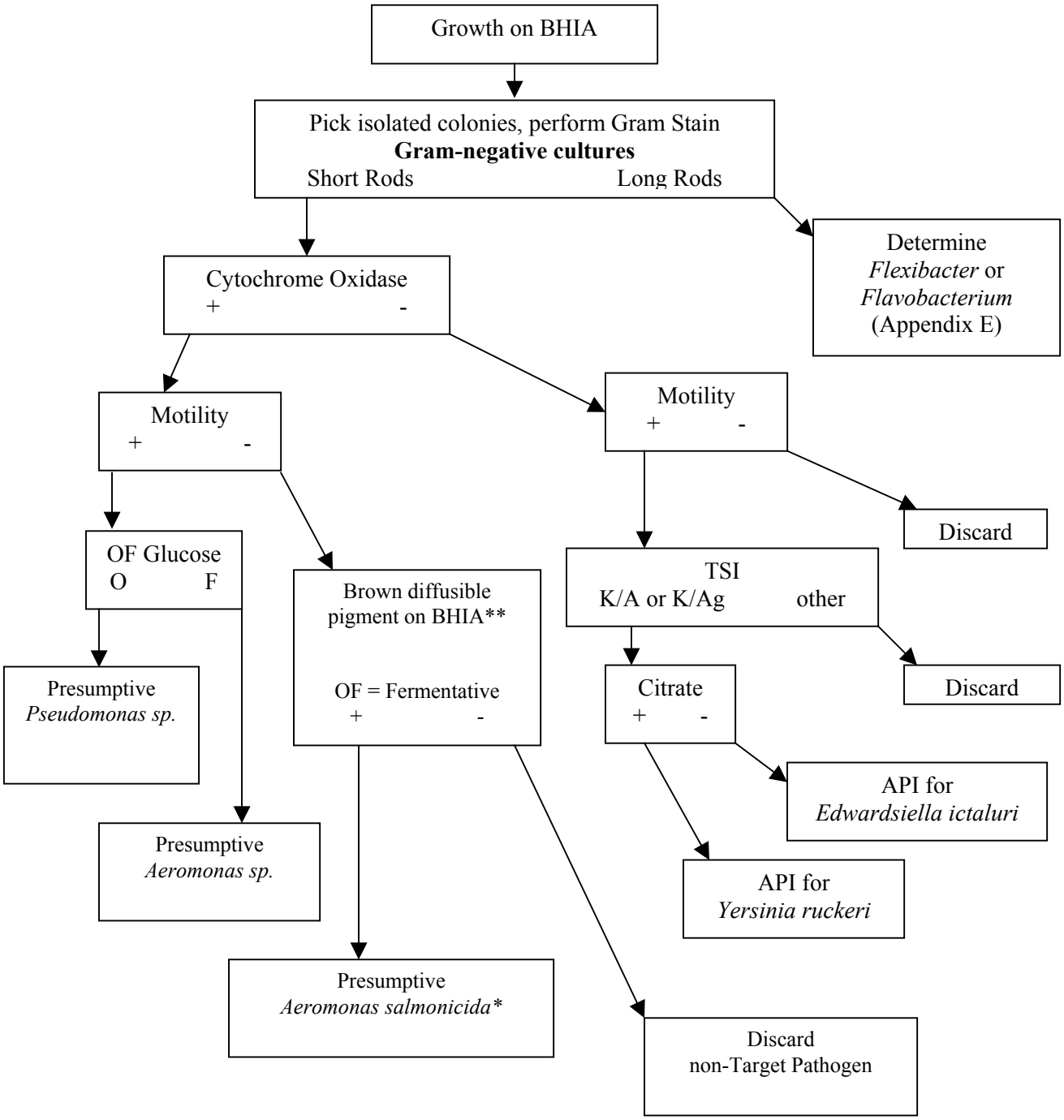
Toranzo, A.E., Y.Santos, T.P. Nieto and J.L. Barja. 1986. Evaluation of different assay systems for identification of environmental Aeromonas strains. Appl. Environ. Micorbiol., 51:652-656.

Appendix D - Flowchart for Targeted Gram-Negative Fish Pathogens



*An Oxidase-negative *A.salmonicida* has been reported (Chapman *et al.,* 1991)
** Some strains of *A.salmonicida* do not produce brown diffusible pigment, or pigment production is delayed.

B. GENERAL QUALITY CONTROL CONSIDERATIONS

1. **Wear gloves and change gloves often.**
   This prevents contamination of sample DNA with degrading nucleases and acids that naturally occur on the skin. Frequent changing of gloves also prevents DNA contamination of hands and work surfaces in the laboratory. Steps in this protocol that are underlined indicate significant sources of error and/or potential for contamination should deviation from this protocol occur.
2. Utilize microcentrifuge tubes with locking caps. Heating of extraction solutions causes unlocked caps to pop open, which can cause release of aerosols that can cause cross-contamination between samples and controls.
3. Always run positive *Renibacterium salmoninarum (Rs)* control tissues as well as negative controls (water, or known negative tissue) from the start of the extraction process through nested amplification to final electrophoresis. This is the only means of assuring validity of the assay and its results.

C. PROCEDURE

1. **Weigh 25-50 mg of kidney tissue** into a 1.5 ml Micro centrifuge tube. Tissue can be fresh, previously frozen, or processed ELISA tissue (pellet).

2. Add **180 µl of Lysozyme Lysis Buffer (LLB)**.

3. Incubate at 37°C for 1 hour, vortexing occasionally.

4. Add **25µl of Proteinase K stock solution and 200 µl of buffer AL.**

5. Mix by vortexing and incubate at 55°C for 30 minutes (vortex occasionally during this incubation period).

6. Incubate at 95°C for another 10 minutes.

7. Add **210µl of ethanol**, mix thoroughly by vortexing.

8. Place a QIAamp spin column in the 2ml collection tube provided. Pipet sample mixture onto the filter in the spin column being careful not to moisten the rim of the column. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1minute. (All centrifugation is at ambient temperature).

9. Transfer the spin column (upper section of tube with filter) into a clean 2 ml collection tube and discard lower tube containing the filtrate.

10. Carefully open the spin column and add **500µl Buffer AW** (wash buffer). Centrifuge as above.

Appendix E – Some Characteristics of Long Gram-negative Bacteria

Flavobacterium columnare	*Flavobacterium psychrophilum*
Morphology and Growth Characteristics	
Growth on BHIA or TSA.	Scant or no growth on BHIA or TSA
Growth at 30°C.	No growth at 30°C; optimum growth at 15-18C
Yellow dry colonies, rhizoid margin, spreading, adhere to agar.	Moist yellow colonies, entire margin, spreading slowly.
Thin gram-negative rods 0.4 x 2-20um	Thin rods, 0.5 x 2-7um
Motility by gliding, or flexing motion: forms "haystacks" of aggregate cells on wetmount	Gliding motility

Biochemical Reactions

No acid from simple or complex carbohydrates (glucose, lactose, galactose and sucrose)

Does not reduce nitrates, produces hydrogen sulfide

Weak catalase positive

Proteolytic for gelatin, casein, albumin

Flexirubin pigments present; colonies turn orange-brown on addition of 20% KOH.

Corroboration Test

Serum agglutination with specific antisera